Biochimie 94 (2012) 132–136

Contents lists available at SciVerse ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Active site mutants of human secreted Group IIA Phospholipase A_2 lacking hydrolytic activity retain their bactericidal effect

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ARTICLE INFO

Article history:
Received 27 July 2011
Accepted 29 September 2011
Available online 6 October 2011

Keywords:
Ca^{2+}-independent
Membrane damage
Active site mutant

ABSTRACT

The Human Secreted Group IIA Phospholipase A_2 (hsPLA2GIIA) presents potent bactericidal activity, and is considered to contribute to the acute-phase immune response. Hydrolysis of inner membrane phospholipids is suggested to underlie the bactericidal activity, and we have evaluated this proposal by comparing catalytic activity with bactericidal and liposome membrane damaging effects of the G30S, H48Q and D49K hsPLA2GIIA mutants. All mutants showed severely impaired hydrolytic activities against mixed DOPC:DOPG liposome membranes, however the bactericidal effect against *Micrococcus luteus* was less affected, with 50% killing at concentrations of 1, 3, 7 and 9 μg/mL for the wild-type, D49K, H48Q and G30S mutants respectively. Furthermore, all proteins showed Ca^{2+}-independent damaging activity against liposome membranes demonstrating that in addition to the hydrolysis-dependent membrane damage, the hsPLA2GIIA presents a mechanism for permeabilization of phospholipid bilayers that is independent of catalytic activity, which may play a role in the bactericidal function of the protein

Phospholipases A2 (PLA2, EC 3.1.1.4) are involved in a range of functional roles involving the release of lipid mediators and membrane remodeling. The PLA2 superfamily currently recognizes 15 groups, and a total of 22 PLA2 isoforms and PLA2-like domains have been identified from sequence analysis of the human genome [1]. These include representatives of PLA2 Groups I, II, III, IV, V, VI, VII, X and XII, and ongoing efforts are focused on the biochemical characterization and physiological functions of these enzymes. The human secreted Group II PLA2s (hsPLA2GII) include IIA, IIC, IID, IIE, and IIF, of which sPLA2-IIC is likely to be a pseudogene [2], and with the exception of hsPLA2GIIA the biological functions of the remaining proteins remain to be clearly defined. The hsPLA2GIIA is widely regarded as a pro-inflammatory factor [3,4], a view supported by recent gene knockout data from mice [5], and altered levels of the sPLA2-GIIA are observed in many pathological situations including cancer [6], respiratory allergy [7] and apoptosis [8]. Physiological roles of the hsPLA2GIIA include a potent bactericidal activity primarily against Gram-positive bacteria, which has been suggested

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doi:10.1016/j.biochi.2011.09.027

l cell wall and cause the hydrolysis of plasma membrane phospholipids resulting in the loss of bilayer integrity and cell death [9–11].

All PLA2s catalyze the hydrolysis the *sn*-2 acyl bonds of *sn*-3 glycerophospholipids [12], and the catalytic cycle of Group I/II/V PLA2s involves activation of a conserved water molecule in the active site by the H48/D99 pair, followed by nucleophilic attack on the *sn*-2 position of the phospholipid to form a tetrahedral reaction intermediate. The Ca^{2+} ion cofactor binds to the D49 carboxyl group and main chain carbonyl oxygens of the calcium binding loop residues 28–33, and is essential both for stabilization of the tetrahedral intermediate and completion of the reaction cycle to form the fatty acid and lysophospholipid products [13,14]. Previous studies have demonstrated that site directed mutagenesis of the active site residues H48 and D49 results in a drastic reduction [15,16] or even loss of catalytic function in Group I/II PLA2s [17,18]. The lack of hydrolytic activity in the Group II Lys49-PLA2 variants isolated from the venoms of viperid snakes is the result of both the D49K substitution and mutations in the calcium binding loop region [19,20]. Despite their lack of hydrolytic activity, the Lys49-PLA2s nevertheless present potent bactericidal effects [21,22], demonstrating that catalytic hydrolysis of membrane lipids is not obligatory for manifestation of the bactericidal effect. This has prompted us to evaluate the bactericidal effects of active site mutants of the hsPLA2GIIA in which hydrolytic activity has been severely reduced or effectively abolished.

2. Materials and methods

2.1. Sub-cloning and construction of the expression vector for hsPLA2GIIA

A full-length cDNA encoding hsPLA2GIIA (GenBank BC 005919) in vector pDNR-lib was from the I.M.A.G.E Consortium/LNNL (http://image.llnl.gov/, clone ID 4274550). The codons encoding G35 and S36 (5′-GGA TCC-3′) form a *Bam*HI restriction site, which was eliminated by silent site directed mutagenesis (5′-GGA TC*T*-3′, base change in italics) in order to facilitate subsequent cloning steps. The protein-coding region was amplified using forward (5′-CAGGCCCATATG*GCT*TTGGTGAAT-3′) and reverse (5′-TTTCCAGGGATCCGGGGACTC-3′) primers that introduced restriction sites for *Nde*I and *Bam*HI (nucleotides underlined) at the 5′ and 3′ extremities respectively. The forward primer introduced the mutation N1A (nucleotides in italics in the primer sequence), which increases the efficiency of post-translation methionine cleavage by N-terminal methionine peptidase, without altering the catalytic properties of the enzyme [23]. After digestion with *Nde*I and *Bam*HI, the amplified fragment was cloned into the equivalent sites of expression vector pET-3a, and nucleotide sequencing confirmed the expected construct.

Site directed mutagenesis of the hsPLA2GIIA by PCR mutagenesis [24] was used to introduce the single mutations G30S (oligonucleotide 5′-GCCACCCACGCTACAGTGGC-3′ – base changes underlined), H48Q (5′-AGCAACAGTCTTGAGTGACACA-3′) and D49K (5′-GTAGCAACATTTATGAGTGACACA-3′). The mutated protein-coding regions were subcloned into the expression vector pET-3a, and all constructs were fully sequenced to confirm the expected base changes.

2.2. Expression of hsPLA2GIIA as inclusion bodies in E. coli

The hsPLA2GIIA and mutants were expressed in *Escherichia coli* strain BL21(DE3)[pLysS] in the form of inclusion bodies, and the solubilization, refolding and purification of the recombinant hsPLA2GIIA were described previously [25]. The secondary structure of all samples was routinely checked by far ultraviolet circular dichroism, and protein concentration was quantified by comparing the measured ellipticity at 222 nm with a standard curve prepared using known quantities of the hsPLA2GIIA.

2.3. Far ultraviolet circular dichroism (far-UVCD) of recombinant hsPLA2GIIA

The far-UVCD spectra of the hsPLA2GIIA and mutants were collected at a concentration of 1.1 mg mL^{-1} in 20 mM HEPES buffer (pH 7.0) with a Jasco 810 spectropolarimeter (JASCO Corporation, Tokyo, Japan) over wavelengths of 185–250 nm, using 100 μm path length quartz cuvettes and a 4 nm bandwidth at a constant temperature of 298 K. The accumulated average of six protein spectra was corrected by subtraction of the spectra of the equivalent buffer in the absence of protein.

2.4. Phospholipase A2 hydrolytic activity assay

The hydrolytic activity of the hsPLA2GIIA and mutants was quantified by measuring fatty acid binding to intestinal fatty acid binding protein conjugated with the fluorescent probe acrylodan (ADIFAB - Invitrogen-Molecular Probes, [26]). Unilamellar liposomes composed of either a 9:1 or a 1:1 molar ratio of dioleoyl phosphatidylcholine (DOPC – Sigma-Aldrich, St. Louis, MO, USA) and dioleoyl phosphatidylglycerol (DOPG – Sigma-Aldrich, St. Louis, MO, USA) were prepared by reverse phase evaporation, and mixed at a final concentration of 30 μg/mL with 3 μg/mL of ADIFAB in reaction buffer (20 mM Hepes, 20 mM NaCl, 1 mM $CaCl_2$). Wild-type (0.1 μg mL^{-1}) or mutant (30 μg mL^{-1}) hsPLA2GIIA was added to the liposome/ADIFAB mixture, and the fluorescence emission at 505 and 425 nm was measured with an excitation wavelength of 385 nm for 30 min using a Spectronic 8100C spectrofluorometer (Spectronic Instruments, Urbana, IL). The specific activity of the enzyme was determined from analysis of the 505 nm/425 nm signal ratio as previously described [25,26].

2.5. Bactericidal activity by counting colony forming units (CFU)

The bactericidal effect of hsPLA2GIIA and mutants over the concentration range of 0–32 μg/mL was evaluated against the Gram-positive bacteria *Micrococcus luteus* (ATCC 9341) as previously described [25].

2.6. Release of entrapped fluorescent markers from liposomes

Membrane damaging activity was evaluated by the release of the entrapped self-quenching fluorescent dye calcein from liposomes composed of either 1:1 or 9:1 molar ratio DOPC:DOPG. Calcein loaded liposomes were prepared as previously described [27], and proteins (final concentration 4 μg mL^{-1}) and liposomes were mixed to a protein:lipid molar ratio of 1:200. Kinetics of membrane damage were monitored by the increase in fluorescence emission at 520 nm with excitation at 490 nm using a Spectronic 8100C spectrofluorometer (Spectronic Instruments, Urbana, IL), and the signal after 140 s exposure to the proteins was expressed as the percentage of total calcein liberation on addition of 5% Triton X-100. Statistical analyses were performed with one-way ANOVA followed by the Tukey comparison test using Origin software (OriginLab, Northampton, MA, USA). A P value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Site directed mutants in the active site region retain native-like structure

The solubilization and refolding of recombinant hsPLA2GIIA in the presence of a gel filtration matrix has been previously used to successfully refold the Lys49-PLA2 bothropstoxin-I [28] and the human secreted group IID PLA2s (hsPLA2GIID [29]). Fig. 1 presents the far-UVCD spectra of the wild-type hsPLA2GIIA and active site mutants G30S, H48Q and D49K. All spectra demonstrate minima at 209 and 222 nm, and a maximum at 194 nm, typical of proteins rich in α-helices and are consistent with the secondary structure content of the wild-type protein as predicted from the crystal structure [30]. The spectral profiles suggest that the secondary structure of the protein was not significantly influenced by mutagenesis in the active site region, which is in accord with crystal structures of the H48Q mutant of hsPLA2GIIA [31] and active site mutants of bovine pancreatic PLA2 [32] where the structural perturbations were limited to the immediate amino-acid neighbors of the mutated residues.

3.2. Catalytic activity is drastically reduced in the active site mutants

The hydrolytic activities of the wild-type hsPLA2GIIA and active site mutants against DOPC:DOPG liposome membranes are presented in Fig. 2. Under these conditions the specific activity of the wild-type protein is 274 μmol min^{-1} mg^{-1}, which is similar to values previously reported against liposome membranes composed of

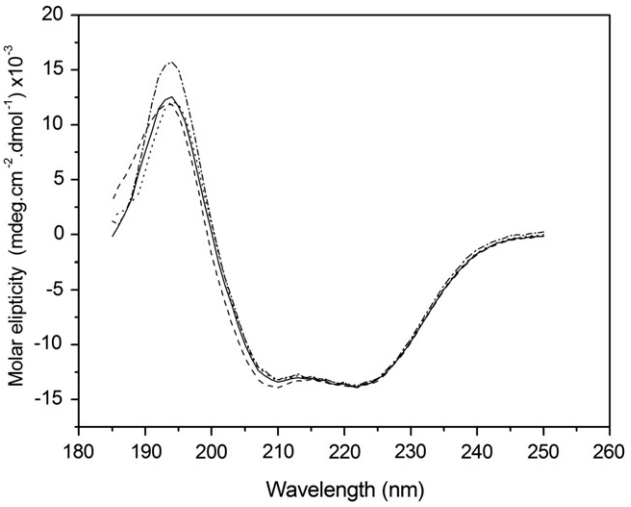

Fig. 1. FarUV CD spectra of wild-type hsPLA2GIIA and mutants. The spectra are from the wild-type (solid line), and the active site mutants G30S (dashed line), H48Q (dotted line) and D49K (dashed-dotted line). See the materials and methods section for further details.

mixtures including anionic phospholipids [31,33]. The active site mutants G30S, H48Q and D49K demonstrate a drastic reduction in catalytic activity, showing 0.02% (for the D49K mutant) and 0.04% (for the G30S and H48Q mutants) of the wild-type activity. No activity was observed in control experiments performed in the presence of EGTA (data not shown), demonstrating that the residual activity of the mutants is dependent on the calcium cofactor. These results confirm previously reports that the H48Q mutants of the hsPLA2GIIA retain low levels of catalytic activity [15,31].

3.3. Bactericidal and catalytic activities are not correlated

It has been previously suggested that the bactericidal activity of the hsPLA2GIIA is the consequence of the hydrolysis of plasma membrane phospholipids, which leads to loss of bilayer integrity and cell death [10,34]. The relation between hydrolytic activity and the killing effect of the protein was evaluated, and Fig. 3 presents the bactericidal effect of the hsPLA2GIIA and mutants against the Gram-positive bacterium *M. luteus*. The wild-type protein causes a 50% reduction in the CFU at a concentration of approximately 1 μg/mL. The corresponding concentrations for the active site mutants are 3 μg/mL (H48Q), 7 μg/mL (D49K) and 9 μg/mL (G30S). These results demonstrate that although the active site mutants show severely reduced hydrolysis they nevertheless retain significant bactericidal activity. It is possible that the residual activity of the active site mutants may be sufficient to cause the observed bactericidal effects, however it has previously been demonstrated that *M. luteus* has a robust response to the hydrolytic challenge imposed by treatment with the hsPLA2GIIA, where hydrolysis of up to 50% of the plasma membrane phospholipids has no lethal effects [35]. Furthermore, our results demonstrate that the levels of activity of the active site mutants will not result in the significant levels of hydrolysis of the plasma membrane phospholipids that were observed with the native enzyme. This suggests that the bactericidal effect of the hsPLA2GIIA is only partly dependent on the hydrolytic function, which in turn indicates that hydrolysis-independent effects may be important for manifestation of the bactericidal activity of the hsPLA2GIIA.

3.4. Liposome membrane disruption of hsPLA2GIIA and mutants is Ca^{2+}-independent

The Ca^{2+}-independent membrane permeabilization of liposome membranes by hsPLA2GIIA was measured by the release of an entrapped self-quenching dye, where loss of liposome membrane integrity results in dilution of the fluorophore with a consequent increase in the fluorescence signal [27,36]. Fig. 4 shows the release of entrapped calcein from liposomes composed of 10% and 50% negatively charged phospholipids after exposure to the wild-type hsPLA2GIIA and mutants in the absence of Ca^{2+}. In Fig. 4A, the release of calcein from liposomes composed of a 9:1 molar ratio of DOPC:DOPG is between 15% and 20% of the total encapsulated marker. In comparison, Fig. 4B shows the release of 40–55% entrapped marker from liposomes composed of an equimolar mixture of the same phospholipids. The bacterial inner membrane is rich in negatively charged phospholipids, and these results indicate that such membranes are susceptible to efficient

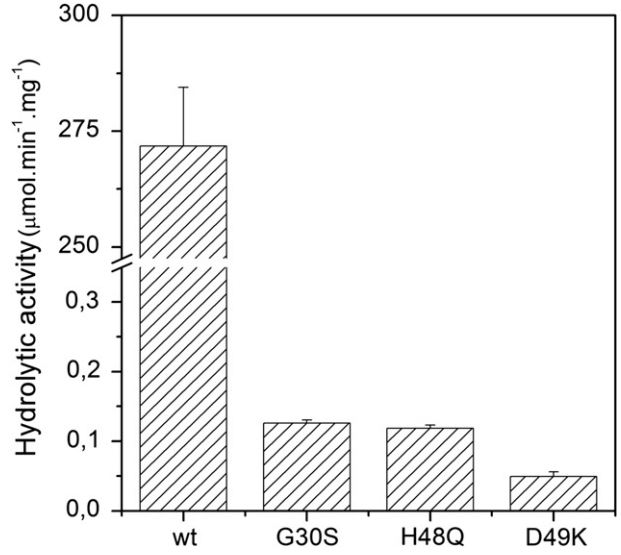

Fig. 2. Hydrolytic activity of the hsPLA2GIIA and mutants against liposome membranes composed of an equimolar mixture of DOPC and DOPG as estimated by fatty acid release detected using ADIFAB. See materials and methods for further details.

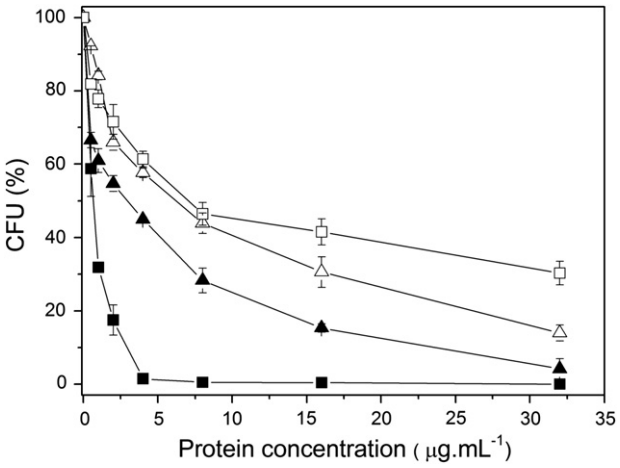

Fig. 3. Bactericidal effect of the hsPLA2GIIA and mutants. The bactericidal effect was measured against *Micrococcus luteus* by the reduction in the number of colony forming units (CFU) with the wild-type protein (closed squares), and the active site mutants G30S (open squares), H48Q (solid triangles) and D49K (open triangles). See materials and methods for further experimental details.

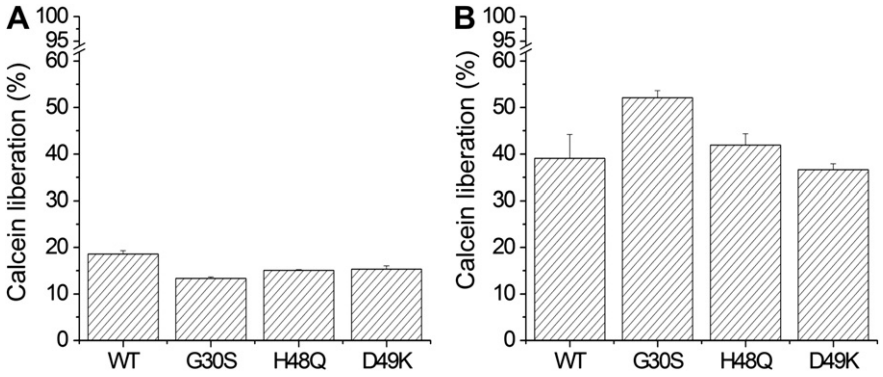

Fig. 4. Calcium independent membrane damaging activity of the hsPLA2GIIA and mutants. Release of the fluorescent marker calcein was measured from liposomes comprised of DOPC and DOPG in 9:1 (A) or 1:1 (B) molar ratios. Figures present the mean +/− standard deviation ($n = 3$) for independent marker release experiments with the wild-type and mutant proteins against liposomes composed of 9:1 (A) or a 1:1 (B) molar ratios of DOPC:DOPG. All experiments were performed using a protein concentration of 4 μg/mL and a protein:lipid molar ratio of 1:200.

permeabilization by the hsPLA2GIIA. The permeabilization of membranes composed of both 10% and 50% negatively charged phospholipids is observed in the absence of the Ca^{2+}-cofactor, demonstrating that the membrane permeabilization effect is independent of hydrolytic activity. Furthermore, the levels of membrane damaging activity of the H48Q and D49K mutants are similar to those observed for the wild-type protein, and in the case of activity against 50% negatively charged membranes, the G30S shows an increased activity in relation to the wild-type protein ($P < 0.05$). These results clearly indicate that mutagenesis of key residues in the active site region do not impair the Ca^{2+}-independent membrane damaging activity of the protein. These results may be compared with the recent demonstration that both the hsPLA2GV and hsPLA2GIIF provoke rapid Ca^{2+}-independent membrane permeabilization against liposome membranes [36], and where the H47Q mutant of the hsPLA2GIIF (analogous to the H48Q in the PLA2GIIA) maintains the same level of activity as the wild-type protein [36].

4. Discussion

The interfacial recognition site [37] or i-face [38] is a conserved structural feature in all group I/II PLA2s, and is comprised of a ring of polar and charged residues surrounding the hydrophobic substrate binding cleft and the active site, resulting in a high affinity of the hsPLA2GIIA for anionic membranes [39,40]. It has been proposed that the bactericidal activity of hsPLA2GIIA against *M. luteus* results from the hydrolysis of anionic phospholipids in the bacterial plasma membrane, an event that is possible only after penetration of the cell wall of the bacteria by the protein [10,34]. Passage of the hsPLA2GIIA through the bacterial cell wall was suggested to involve the continuous formation and breaking of multiple electrostatic interactions between cationic amino-acid side-chains and anionic cell wall components [9,34], and this model has been used to explain the reduced bactericidal effects of charge reversal mutants of the protein [9,41].

This model of hsPLA2GIIA bactericidal activity emphases the membrane damage due to phospholipid hydrolysis. However, hydrolysis-independent bactericidal effects are observed in the Group II Lys49-PLA2s from the venoms of viperid snakes [21,22]. The current knowledge as to the killing mechanism of these catalytically inactive Lys49-PLA2s against Gram-negative bacteria shares several features with the proposed mechanism for the hsPLA2GIIA. Using recombinant bothropstoxin-I, a Lys49-PLA2 from the venom of *Bothrops jararacussu*, it was demonstrated that protein is able to cross the bacterial cell wall and disrupt the integrity of the phospholipids in the plasma membrane [21]. Furthermore, alterations in the permeabilization of the bacterial plasma membrane by Lys49-PLA2 mutants have been shown to correlate well with the membrane destabilizing effect against liposome membranes composed of anionic phospholipids [21]. These results have led to the suggestion that the permeabilization of the bacterial plasma membrane and liposome phospholipids bilayers by the Lys49-PLA2s occurs by a related Ca^{2+}-independent mechanism of action. A “micelle nucleation” model for this activity has been proposed [42,43], whereupon association with the target membrane a sufficient number of phospholipids bind to the IRS of the protein to form a pre-micelle PLA2/phospholipid complex. This complex dissociates from the membrane thereby removing phospholipids and resulting in the loss of bilayer integrity. Given that both the Lys49-PLA2s and the hsPLA2GIIA present a high pI and have interfacial recognition sites that are rich in cationic residues, it is reasonable to propose a conserved Ca^{2+}-independent membrane damaging mechanism for both the Lys49-PLA2 and the PLA2GIIA.

A Ca^{2+}-independent function for hsPLA2GIIA has recently been described in cultured THP-1 cells differentiated to a macrophage-like cell phenotype, where the protein is involved in the transport of extracellular anionic amphiphiles (presumed to be cell debris) to the cell nuclei [44]. It is proposed that these anionic amphiphiles are transported in the form of a complex with the protein [44], which shares striking similarity with the idea of accumulation of amphiphilic molecules at the IRS of the protein by a micelle nucleation mechanism.

Data is accumulating which therefore indicates that physiological functions of the hsPLA2GIIA may be independent of hydrolytic activity, and here we have presented evidence indicating that bacterial killing by the protein is the result of hydrolysis-dependent and hydrolysis-independent mechanisms acting in conjunction. Bactericidal effects due to the perturbation of the bacterial plasma membrane by non-hydrolytic mechanisms are well established for cationic antibiotic peptides [45], and the results presented here suggest that non-hydrolytic mechanisms may also be relevant for the activity of the hsPLA2GIIA. Further studies are needed to evaluate the physiological relevance of this Ca^{2+}-independent mechanism both in the hsPLA2GIIA and in other PLA2s with a high density of cationic residues in their interface recognition sites.

Acknowledgments

The financial support of CNPq (476415/2006-3) is acknowledged. EAA, TFL and LC were recipients of FAPESP fellowships 05/

50379-0, 02/12746-2 and 01/00279-8. We are grateful to Prof. Dr. Claudio Tedesco (FFCLRP-USP) for use of the spectrofluorimeter.

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